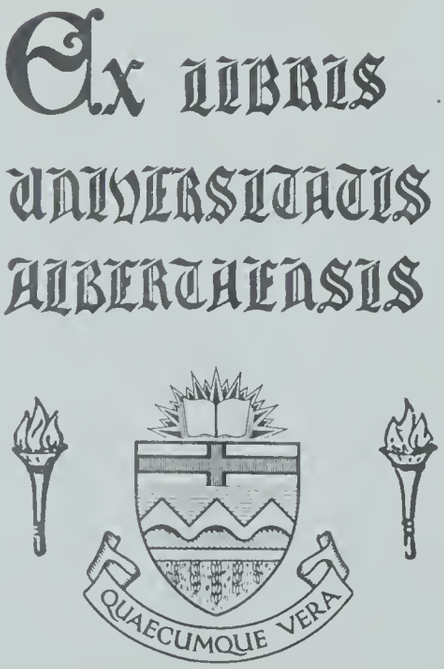

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LISSAMINE GREEN STAINING OF THE CORNEAL ENDOTHELIUM AND EVIDENCE FOR A GLYCOPROTEIN COAT ON ITS FREE SURFACE

by

© ANGUS H. KIRK

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled LISSAMINE GREEN STAINING OF THE CORNEAL ENDOTHELIUM AND EVIDENCE FOR A GLYCOPROTEIN COAT ON ITS FREE SURFACE, submitted by Angus H. Kirk, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

In the search for a simple, pre-operative screening test of the viability of eye bank corneas, the most promising method has been supravital staining of the corneal endothelium with Lissamine green (L.G.), a non-toxic dye which penetrates dead cells only.

This study was designed to assess the reliability of L.G. staining as an indicator of the viability of cat corneas after varying intervals of storage by correlating the number of endothelial cells stained by L.G. with the degree of temperature reversal -- the reversal, by heat, of cold-induced stromal swelling, a process believed by many to be an indicator of corneal viability.

Corneal thickness was measured immediately after enucleation, after storage at 4^{o}C, and after incubation at 37^{o}C, and the percentage decrease in thickness after incubation was calculated. Lissamine green was then applied to the endothelium and the number of stained cells counted.

As expected, temperature reversal declined as storage time was increased. Although the number of dead cells stained with L.G. had been shown to increase with storage of up to 48 hours (Jans 1968), further storage of up to 120 hours produced no further increase in cells stained. Though it indicates that L.G. staining is not a useful test of corneal viability, this was the first of three unexpected findings which led to the hypothesis that the endothelial surface is covered by a glycoprotein surface coating. The second was that stained cells were usually found in groups overlying folds,

suggesting that they were exposed to the stain where a surface layer was parted by trauma. The third finding was the application of hypotonic saline to the endothelial surface caused the appearance of a mosaic of refractile hyaline granules with properties of glycoprotein.

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INTRODUCTION

In view of the great variation in conditions to which donor eyes are exposed, and the great variation in individual tissues, there is a great need for a rapid, reliable test for corneal viability to replace the present reliance upon time elapsed since death or enucleation. Since experience has shown that the integrity of the endothelium is essential to successful penetrating keratoplasty, investigation has focussed upon tests for the viability of the endothelium.

The most promising approach to assessment of endothelial viability is the use of non-toxic supravital stains. Lissamine green, while remarkably non-toxic (Goldacre 1959) consistently and rapidly penetrates irreversibly damaged cells, while viable cells remain impermeable (Holmberg 1961). Jans and Hassard (1969) recently used lissamine green as a test of the viability of the endothelium of cat corneas stored under eye bank conditions for up to 50 hours. They found a consistent correlation between the number of stained cells and both the storage time and the degree of temperature reversal -- the degree to which warming reverses cold-induced swelling of the cornea.

The original purpose of the present study was to use Jans and Hassard's method of lissamine green staining to determine the number of dead cells in the endothelium of cat corneas stored beyond 50 hours. The number of stained cells was then correlated with the temperature reversal effect, an effect thought to reflect corneal

viability. A cornea cooled to 4°C in a moist atmosphere imbibes water and swells; when this cornea is warmed to body temperature, reversal of the swelling occurs, possibly because of reactivation of corneal metabolism, and the cornea returns to near-normal thickness.

Several unexpected findings, including the observation that the number of stained cells failed to increase with storage times of up to 120 hours, led to the hypothesis that the endothelial surface is covered by a glycoprotein surface coat, to a second line of investigation designed to characterize this free surface coat in terms of its reactions to chemical and physical agents.

MAINTENANCE OF CORNEAL HYDRATION

Temperature reversal effect is the term applied by Davson (1955) to the reversal of cold-induced swelling of the cornea by rewarming the cornea to body temperature. In this study, the degree of temperature reversal, which has been widely regarded as a measure of corneal viability, was correlated with the degree of staining with lissamine green (L.G.) as a test of the validity of L.G. staining in assessing corneal viability. It is first necessary, therefore, to consider what is known of the factors involved in producing and maintaining deturgesence of the cornea.

STRUCTURAL FACTORS CONTRIBUTING TO STROMAL SWELLING

Histological and chemical structure

The marked swelling tendency of the stroma is due to its structure and chemical composition. The lattice-like and unwoven arrangement of the collagen fibrils offers very little restraint to rapid swelling. The ground substance consists of a complex of protein with three polysaccharides: keritin sulfate, chondroitin sulfate, and chondroitin. These acidic polysaccharides have many fixed anionic groups which, unless shielded by cations such as sodium, tend to bind and retain water. The importance of water-binding by stromal polysaccharides was demonstrated by Hedbys (1961) who showed that corneal swelling can be inhibited by pre-treatment with a quaternary ammonium base which precipitates the polysaccharides.

He also showed that hydrolysis of ground substance polysaccharides by hyaluronidase markedly decreased the swelling tendency. Electron micrographs by Francois et al (1954) showed that the diameter of collagen fibrils remained unchanged during corneal swelling at physiological pH.

Swelling forces

Dohlman (1957) demonstrated that in vitro swelling of the cornea can be prevented by increasing the colloid osmotic pressure of the bathing solution to approximately 2½ times that of serum. Further increases in colloidal osmotic pressure caused greater than normal dehydration of the stroma. Using a transducer, Hedbys and Dohlman (1963) found a swelling pressure of 60 mmHg at normal corneal hydration. This is close to the value obtained by Dohlman using hyperosmotic dextran. They observed that the swelling pressure increased markedly when the stroma was dehydrated and fell to zero when it became hydrated to about twice the normal level.

Reasoning that the forces which counteract the swelling pressure in the stroma must produce a "suction" pressure, Hedbys et al (1963) inserted a small cannula into the stroma and measured a negative imbibition pressure which, in vitro, was equal but opposite in sign to the swelling pressure. In vivo, the imbibition pressure was lower than the swelling pressure by a factor equivalent to the intraocular pressure.

The swelling potential of the stroma is such that isolated pieces of stroma will hydrate to at least four times the normal level.

The probable reason why the swelling and imbibition pressures in the chilled intact eye fall to zero at approximately twice the normal hydration is a structural restraint imposed when an intact cornea maintains its normal scleral relationship.

METABOLIC CONTROL OF HYDRATION

Several passive factors contribute to the control of corneal hydration, including the effect of the endothelium and epithelium as barriers to the inward diffusion of adjacent fluid, and the effect of evaporation from the epithelial surface. (Dohlman et al, 1966)

About 15 years ago, the first evidence for an active, metabolic control of corneal hydration was provided by studies of the temperature-reversal effect -- the reversal of cold-induced corneal swelling by warming the cornea. It had been known that when eyes were cooled in a moist chamber, the cornea would swell. Davson (1955) found, however, that when a chilled eye was warmed to body temperature, the cornea returned to an almost normal level of deturgesence. It was found that this temperature reversal effect required an oxygen supply and was reduced or abolished by certain metabolic poisons (Harris, 1957, 1960).

At present, the mechanism whereby metabolism maintains normal hydration is in hot dispute. The two theories are: (i) the endothelium has an active transport mechanism pumping fluid from the cornea; (ii) sodium pumped into the stroma by the epithelium maintains the cohesive forces that restrict the swelling of the

acidic stromal polysaccharide-protein complexes.

Theory of outward active transport

It has long been known that if either the endothelium or epithelium is damaged, the cornea rapidly swells. Because damage to the endothelium results in much more extensive stromal edema than does epithelial damage, it has long been assumed that the endothelium is the primary site at which water is removed from the cornea.

Unfortunately, all efforts to identify an active transport mechanism for the outward movement of fluid or sodium by the endothelium or the epithelium have failed. On the contrary, metabolic poisons such as dinitrophenol and ouabain failed to block deturgesence at concentrations sufficient to inhibit cation transport mechanisms in other tissues (Langham and Taylor, 1956, Brown and Hedbys, 1965). Whereas lithium inhibits active transport mechanisms in other tissues, where sodium is the usual ion transported, replacement of over 50% of the corneal sodium by lithium failed to inhibit deturgesence of the swollen cornea. (Schon, 1957). In addition, the endothelium shows no electrical potential, a feature of most cell layers having an active transport mechanism, and no evidence has been found from isotope studies for active transport across the endothelium (Green, 1967).

Pinocytotic activity has been demonstrated in the endothelium (Donn et al, 1961, Kaye et al, 1962), and the possibility remains that this could constitute a mechanism for the active transport of fluid from the stroma.

Theory of inward transport of sodium to maintain stromal cohesive forces

Although no transport mechanism has been demonstrated for the movement of fluid or sodium outward from the stroma, Donn and associates (1959) demonstrated that the epithelium has an active transport mechanism for moving sodium into the stroma. The significance of this mechanism was unknown till 1961 when, after demonstrating that the polysaccharide-protein ground substance was the site of water uptake in stromal swelling, Hedbys proposed that stromal swelling results from repulsive forces between fixed anionic charged groups in the acidic polysaccharide chains. It now seems logical to regard sodium as the major counterion which, by shielding the fixed negative charges on the polysaccharide chains, decreases their rigidity and mutual repulsion, and allows them to assume their more compact, coiled form.

Since it has been demonstrated that the aqueous humor and stromal solution are isosmotic (Brubaker et al, 1962), Otori's finding (1967) that the concentration of sodium in the normal cornea exceeded that in the aqueous suggests that there may be interaction between this cation and the acidic polysaccharides. The excess sodium concentration apparently does not contribute to the total stromal osmotic pressure, and hence may be assumed to be bound to the polysaccharide.

While varying the sodium concentration of the solution bathing the epithelium, Green (1962) found a straight-line relationship between corneal thickness and the rate of sodium transport, suggesting

that the control of corneal thickness is dependent on the inward sodium transport rate. The normal level of bound sodium has been calculated to be 28-30 mM above the concentrations of the free stromal and endothelial bathing solutions (Green, 1969). There is thus an osmotic gradient from the polysaccharide to the free solution. At a steady state hydration, the rate of supply of sodium by the epithelium must equal the rate of loss across the endothelium.

Significance of the temperature reversal effect

There is now some question about the mechanism of the temperature reversal effect. It was initially assumed to reflect reactivation of an active transport mechanism in the corneal endothelium, and this remains a possible explanation. It is puzzling, however, that this effect was produced in pig eyes stored for up to 6 days (Lavergne 1963). Furthermore, although the temperature reversal effect has been produced in corneas after the epithelium has been removed and the stromal surface exposed to moist air (Mishima and Kudo 1967) or to oil (Dikstein and Maurice 1968), no published attempt has been made to produce this effect after removal of the endothelium.

In the course of investigating the properties of hydrophilic soft contact lens material, it was observed by Itoi (1965) that when lenses of polyelectrolyte gel made from cross-linked polymers were taken from boiling water and placed in cool water, they showed a temperature reversal effect and swelled further. Since the ground substance of the cornea consists of a polysaccharide-protein gel with

repulsive forces between fixed charges on the acidic polysaccharide chains, Itori's findings suggest that the temperature reversal effect is a physico-chemical characteristic of polyelectrolyte gels

The ionic state of the corneal ground substance is, of course, influenced by the barrier function and metabolic activity of the epithelium and endothelium, and the fact remains that damage to the endothelium results in rapid hydration of the cornea (Harris 1957), and presumably loss of the temperature reversal effect -- a postulate which should be investigated. Hence, the temperature reversal may represent a measure of endothelial viability.

ASSESSMENT OF CORNEAL ENDOTHELIAL VIABILITY

Numerous approaches have been developed to the assessment of the frequency of cell death. The most useful tests of cell viability are:

1. Microscopic appearance of cells
2. Time-lapse cinemicrography
3. Respiratory activity
4. Enzyme assays
5. In vitro tissue cultures
6. Reinnoculation of ascites tumor cells
7. Differential penetration of dyes.

Of these 7 types of viability test, only one shows promise as a rapid, practical means of preoperative assessment of the condition of the endothelium of donor corneas. This is differential staining of dead cells by a non-toxic supravital dye. All of the 7 above-mentioned methods have, however, been used by Holmberg (1961) in coordinated, comparative studies to demonstrate the validity and reliability of staining with the non-toxic dye, lissamine green (L.G.), as an indicator of cell death.

CORRELATIVE SUPRAVITAL STAIN STUDIES

Holmberg (1961) first compared results of the differential staining with lissamine green with those of other supravital dyes which have been reported as screening tests for cell death. He found that consistent and reproducible differential counts were obtained in cell suspensions with L.G. and trypan blue. Slightly higher differential counts were often obtained with nigrosin and

erythrosin B. Slightly differing results were also obtained with Eosin Y because living cells frequently developed a pink hue difficult to distinguish from the positively red-stained cells. Added to cell suspensions treated with dilute solutions of any of the above dyes, however, L.G. penetrated only those cells already stained. Joint results of the dye, metabolic, and other studies indicate that undamaged cells are impermeable to L.G. and the other above-named dyes.

The most significant finding in Holmberg's comparative supravital stain studies is the consistency of the correlation between staining with L.G. and trypan blue. Several studies have demonstrated that trypan blue penetrates only cells which are dead or severely injured (Evans and Schulemann 1914).

After demonstrating a rough correlation between supravital para-nitroblue tetrazolium (PNBT) staining and differential tissue culture using corneal endothelium, Stocker (1966) reported that on the corneal endothelium, trypan blue staining correlated fairly well with PNBT staining.

CORRELATION OF METABOLIC STUDIES WITH SUPRAVITAL STAINING

Respiratory rate measurements on ascites cell populations containing varying ratios of injured cells showed a proportionality between the decline in oxygen consumption and the number of L.G.-permeable cells, as well as a decrease in the average per-cell enzyme activity paralleling the degree of L.G. staining in the cell population (Holmberg 1961).

Correlation of morphology with supravital staining

In all cells showing the cytological changes characteristic of irreversible cell injury, L.G. instantly penetrated the cytoplasm and nucleus. Time-lapse cinemicrography revealed that all cells permeable to L.G. had complete lack of membrane activity, while the non-permeable cells showed vigorous surface activity. Most non-permeable cells eventually became adherent to the glass surfaces, while all permeable cells remained rounded and free-floating (Holmberg 1961).

The foregoing studies indicate that L.G. staining is a rapid, reliable means of differentiating viable from irreversibly damaged cells.

EXTERNAL COATS OF THE PLASMALEMMA (THE GLYCOCALYX)

Many types of mammalian cells have a surface coating rich in polysaccharides. Examples are the coatings of the basal, lateral and free surfaces -- the "basement membrane," "intercellular cement," and surface coat, respectively -- demonstrated by electron microscopy and immunochemical techniques on a wide variety of cells. The ability to elaborate and deposit surface coatings of polysaccharide-protein substances is such a common property of cells that the question has been raised whether such a coating is a general feature of all cells. The term external lamina or glycocalyx has been proposed for the extracellular polysaccharide-rich coating investing all surfaces of cells. (Bennett, 1963). This concept may be useful provided it does not imply that all the surface coatings of cells are similar in origin, composition, properties, or relation to the underlying cell membrane, even on different areas of the same cell.

PROPERTIES OF "MUCOPOLYSACCHARIDES"

NOMENCLATURE AND CLASSIFICATION:

In describing the chemical nature of extraneous cell coats, most morphologists still use the general term "mucopolysaccharide," apparently because these coats stain with Periodic Acid Schiff and other techniques specific for a large group of amino sugar-containing polysaccharide and polysaccharide-protein substances now known to consist of two major classes of compound, only one of which, the glycoproteins, is now believed to be an important component of the glycocalyx.

There is great confusion in the nomenclature of amino sugar-containing polysaccharides with each author defining mucopolysaccharide and mucoprotein in his own way. Thus, the prefix "muco" is used to designate either the sugar, or amino sugar, or polyaminosaccharide, or protein moiety of a polysaccharide-protein complex. Since mucus is a physiological rather than a biochemical term, Jeanloz (1960) has proposed that the prefix "muco" be dropped from the chemical nomenclature.

The nomenclature of purified polysaccharides is covered by rules of the American Chemical Society. Thus, the group commonly known as "acidic mucopolysaccharides" (e.g. hyaluronic acid, chondritin sulfates and other compounds containing two or more different monosaccharides with a uronic acid and a hexosamine component) is called "glycosaminoglycuronoglycans." In vivo, however, all polysaccharides are complexed with protein.

Janloz (1960) divided polysaccharide-protein compounds into two groups: (i) those having the carbohydrate and polypeptide components attached through "weak" electrostatic links (salt or ionic linkages, hydrogen bonds, etc.); these he termed polysaccharide-protein complexes (e.g. chondritin sulfate-protein complex); (ii) those having the carbohydrate and polypeptide components attached by a "strong" covalent link; these he termed glycoproteins. The first group includes the "acid mucopolysaccharides", and the second group, the "neutral" blood group and plasma glycoproteins, gonadotropins, orosomucoid, and epithelial mucous secretions.

There is general agreement upon the division of animal polysaccharide-protein compounds into these two groups. There is, however, recent evidence that in the chondroitin sulfate-protein and heparin-protein complexes there is a portion of the carbohydrate which is covalently bound as well as a portion electrostatically bound. Because classification of the two main groups of carbohydrate-protein compounds on the basis of the type of carbohydrate-protein linkage has thus proved inadequate, Gottschalk (1962) has classified these two groups according to the characteristic structural features of their carbohydrate moieties. In mammalian polysaccharide-protein complexes, the carbohydrate moiety is a linear structure composed of small repeating units and a relatively high number of sugar residues. In some, the polysaccharide-protein linkage appears to be at least partly covalent, but may be electrostatic in others. Glycoproteins, on the other hand, contain a carbohydrate moiety of one or more heterosaccharides with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain.

Molecular structures

Structure typical of a polysaccharide-protein complex:

Most polysaccharide-protein complexes are believed to be rod-like structures. On the basis of light-scattering and viscosity measurements of chondritin sulfate-protein complexes, Partridge et al (1961) and others (for review see Muir, 1961) have concluded that the basic molecular unit is a rod of length 3,700 A and molecular

weight 4.0×10^6. The proposed structure is that of a protein core running the length of the rod, along which are distributed 62 units of chondroitin sulfate of molecular weight 50,000. Part of this huge, complicated complex is combined by electrostatic, and part by covalent bonding. Details of its geometry are still very uncertain. Hyaluronic acid is believed to have a structure more like that typical of the glycoproteins, i.e. a random coil with some degree of stiffness in which mutual repulsion between ionized carboxylic acid groupings appears to cause expansion and stiffening of the coil, especially in solutions of low ionic strength (less than 0.1).

Structure typical of a glycoprotein:

The most widely accepted general model of the glycoprotein molecule is that of a flexible random coil which, depending upon the degree to which it is coiled up or extended, may have a spherical, elliptical, or rod-like domain. The physical rigidity (or steric hindrance of side chains) limits the degree of contraction of the coil, but the amount of interaction between molecule and solvent and between the molecule and itself fixes the average configuration. Flexible polyelectrolytes can self-interact very strongly, and their configuration is both pH and ion-strength dependent. Such a flexible polyelectrolyte, at pH's where the molecule is extensively ionized, will, in the absence of added electrolyte, be very greatly extended and rod-like, due to mutual repulsion of the charges on the flexible chain. For example, in glycoproteins containing large amounts of sialic acid, at pH above 4, where sialic acid is almost completely ionized, the repulsive forces

between adjacent acidic residues will be great unless these are extensively shielded by numerous counter-ions. If the molecule is not rigid, it will become greatly extended by these forces as the ionic strength is reduced. Reversible aggregation between glycoprotein molecules can occur with changes in pH, ionic strength, and even with dilution. A random-coil molecule which assumes an extended or distorted configuration in response to an applied stress is in a statistically unlikely configuration. When the stress is removed, it resumes its more probable coiled form; the molecule may therefore be considered to have elastic properties. At high concentrations, where random-coil molecules, if sufficiently expanded, interpenetrate one another considerably, the macroscopic rheological properties of the network become very elastic.

EVIDENCE FOR GLYCOPROTEIN NATURE OF THE GLYCOCALYX:

Staining specificity:

Rambourg found evidence that phosphotungstic acid is nearly specific for glycoproteins (Groniowski, et al, 1969); with this stain, Pease (1966) demonstrated the surface coat on renal epithelial cells.

Breakdown by neuraminidase:

Colloidal thorium and ruthenium red staining both demonstrate the surface coat on kidney epithelial and capillary endothelial cells. Staining of the cell surface by both techniques was eliminated by treatment of the surfaces with neuraminidase, an enzyme which breaks down carbohydrates containing sialic acids (Groniowski et al 1969).

Extraction of glycoproteins from surface coats:

Glycoprotein fractions have been extracted from the basal lamina of a variety of epithelial cells and have been identified by physicochemical methods (Pierce et al 1964).

STRUCTURE OF THE GLYCOCALYX (EXTERNAL LAMINA)

With improved techniques of tissue preparation for electron microscopy, evidence is rapidly accumulating in support of the concept of a glycoprotein extraneous coat, the glycocalyx, as an almost universal feature of cells. Such an investing layer has been demonstrated not only on a wide variety of epithelial cells, but in many cells of mesenchymal origin, including fibroblasts, smooth and striated muscle cells, pericytes of capillaries, and Schwann cells. Immunochemical methods have demonstrated a polysaccharide-rich surface coat on erythrocytes (Kabat, 1956). There is increasing interest in the extent to which the distinctive physiological characteristics of the plasmalemma on different cells depends upon the properties of their associated glycoprotein.

BASAL LAMINA (BASEMENT MEMBRANE):

At the boundary between epithelium and underlying connective tissue is a continuous glycoprotein layer originally termed the basement membrane. As originally used by light microscopists, however, this term was not limited to the relatively thin, amorphous glycoprotein layer, but included some of the underlying connective tissue. Furthermore, the term "membrane" now generally denotes a specific trilaminar lipoprotein structure of 80 to 120 A. It has been suggested, therefore, that the glycoprotein substratum of epithelia be referred to as the basal lamina.

In electron micrographs of epithelia, the basal lamina is a dense band 500 to 700 A thick separated from the basal cell membrane by a light zone 400 A wide. At high magnification, it has a texture suggesting a dense mat of extremely thin filaments running mainly in the plane of the layer. In the dense layer, individual fibers are difficult to resolve, either because they are so densely compacted, or because they are embedded in an amorphous matrix of similar density. Unlike those in the free surface coat of epithelial cells, the filaments in the basal lamina are thinner and unbranched, and none end in the cell membrane (Fawcett, 1966). The fine filamentous component has been identified as tropocollagen and the amorphous matrix as a glycoprotein. Immunological studies have shown an antigen common to most epithelial basal lamina but distinct from connective tissue components (Pierce et al, 1964). Most evidence now indicates that the basal lamina is not a condensation of the connective tissue ground substance, but a product of the epithelial cells.

FREE SURFACE COAT:

In recent years, with the electron microscope and other techniques, a polysaccharide-rich coating has been demonstrated on the free surface of a wide variety of epithelial and non-epithelial cells. These coats can be divided into those having a filamentous microstructure and those which have an amorphous or granular appearance with few, if any, filaments demonstrable.

Filamentous coats:

The plasmalemma at the free surface of gastrointestinal

epithelial cells has a surface coating consisting of a dense meshwork of delicate branching filaments matted together, having the staining characteristics of a glycoprotein. The early concept that this surface layer was simply an adherent layer of goblet cell mucus was invalidated by recent studies of its histochemical properties and fine structure, which showed it to be a product of the cells it coats, and by the finding that, unlike the basal lamina which forms an uninterrupted substrate for epithelia of mixed types, the free surface layer was invariably present on the chief cells but lacking on parietal cells.

At high magnification, the filaments are 25 to 50 A thick and may extend 0.1 to 0.5 microns from the surface. They appear to branch repeatedly. At their base, they appear to be continuous with the outer leaf of the unit membrane. The filaments extending from adjacent microvilli intermingle to form a network (Fawcett, 1966). This filamentous surface coat is extremely resistant to destruction by enzymes or other chemical or physical agents. Free surface coatings with a similar filamentous microstructure have been demonstrated on a number of other epithelia, including that lining the gall bladder (Yamada, 1955), and the urinary bladder of the toad (Choi, 1965).

Non-filamentous surface coats:

Capillary endothelium:

The Ruthenium red-osmium tetroxide preparation for electron microscopy, which appears to be specific for acidic polymers, has

demonstrated a layer on the luminal surface of the capillary endothelial cell which arises at the outer leaflet of the unit membrane, extends several hundred Angstroms into the vessel lumen, and fades out along a fluffy, indeterminate boundary. Unlike the free surface coat on gastrointestinal epithelia, this endocapillary surface layer has shown no regular structure or periodicity other than flocculent local variations in density and occasional irregular strands.

Luft (1966) observed depressions in the endocapillary layer as if several vesicles had opened, spread out, and become part of the cell membrane, contributing themselves and their contents to the surface coat. In some segments, the density of the layer and of the vesicles beneath showed considerable variation. These observations were considered compatible with the hypothesis that the endocapillary layer is a secretory product of the endothelial cell via the vesicles.

Nephron:

An amorphous ruthenium red positive free surface coat having the same density as, and apparently continuous with the outer leaf of the unit membrane has been demonstrated on the lumenal face of the epithelial cells of the nephron (Groniowski et al, 1969). The slit pores of the glomeruli were also covered by a coat continuous with the surface coat of the adjacent foot processes. The coat lining the microvilli of the proximal convoluted tubules completely filled the intervillous spaces. The same workers demonstrated this

coat by means of the Hale colloidal iron and the colloidal thorium methods and showed that the Hale reaction was abolished by neuraminidase digestion, a finding which indicates the presence in the coat of sialic acid-containing glycoprotein.

Intercellular Polysaccharides

Between the boundaries of adjacent cells there is usually an intercellular space which, in epithelial cells, is 150 to 200 A in width. This cleft is believed to be occupied by a glycoprotein with properties differing from those of the basal lamina or free surface coat. One of these differences is the ease with which this layer is extracted during specimen preparation, and hence, this interspace frequently appears empty. With specimens prepared under favorable conditions, however, this space is occupied by a homogenous material of low density.

The strikingly uniform spacing between adjacent membranes suggests some cohesive force, whether intercellular material or long range physical forces produced by the membrane. There are also specialized sites of firmer attachment between opposing membranes. These are the desmosome or macula adherens, the intermediate junction or zonula adherens, and the tight junction or zonula occludens. In the latter, the cell membranes contact each other and their outer leaflets appear to fuse, completely obliterating the intercellular space and serving as a barrier to intercellular diffusion.

Extension into the intercellular space of the ruthenium red-staining glycoprotein surface coat was observed by Luft (1966), on the capillary endothelium, and by Groniowski et al (1969) on the

renal tubular epithelium. This stain was, however, very slow to pass the zonula occludens.

FUNCTIONS OF THE GLYCOCALYX

Filter

In the intercellular spaces and at the basal lamina, the glycocalyx appears to serve as a filter, retarding the passage of particles, molecules or ions above a certain size. By virtue of filtration and selective binding, a glycoprotein coating around a cell can influence the composition of the environment close to the external surface of the cell. If charged groups are present, the surface coating shows properties of an ion exchange resin.

Selectivity in uptake of substances by pinocytosis

Evidence is accumulating for the suggestion that the surface layer can, by virtue of the chemical nature of the groupings available, produce selective binding of particular classes of ions or molecules to the cell surface prior to its invagination to form pinocytotic vesicles (Bennett, 1963). The most graphic example of this mechanism is seen in the amoeba (Brandt and Pappas, 1960). The plasmalemma is entirely covered by a filamentous surface coat with staining properties of an acidic polysaccharide. Colloidal particles such as thorium dioxide and ferritin are selectively absorbed to the surface coating in high concentration. This is followed by invagination of the heavily encrusted surface to form a pinocytotic vesicle. This process was also demonstrated by Choi (1963) in the toad bladder epithelium.

A similar mechanism is described for the normal uptake of ferritin by cells of the erythropoietic series in mammalian bone marrow (Bessis and Breton-Gorius, 1959; Fawcett, 1963). The cell membrane of the erythroblast has the appearance of a smooth-surfaced unit membrane, but in certain small plaques destined to form pinocytotic vesicles, the plasmalemma develops a visible external coating. These specialized areas show areas of faint vertical striation, suggesting the presence of fine filaments projecting from the outer leaflet of the membrane. Particles of ferritin are found adhering to the outer surface of the filamentous surface coat on these thickened areas, but not to other areas of the cell membrane. Adsorption of ferritin is apparently soon followed by pinocytotic invagination of the ferritin-encrusted area of membrane.

Although electron micrographs have not yet demonstrated a glycoprotein coating on the surface of the corneal endothelium, Kaye and Pappas (1962) found that when thorotrast was injected into the aqueous humor, the particles attached to the endothelial cell surface, and the marker was internalized by pinocytosis. When this marker was injected into the corneal stroma, it was also internalized by the endothelial cell at its basal surface. The same phenomenon is seen in the endothelial cells of capillaries when markers such as thorotrast are injected into the capillary lumen and into the tissue outside the capillary wall. The glycoprotein layer on the surface of the corneal endothelium may thus play an important role in the metabolism of the endothelial cells, and possibly of the underlying stroma.

REPORTED EVIDENCE FOR A FREE SURFACE COAT ON THE CORNEAL ENDOTHELIUM

Using phase contrast microscopy, Preziosi (1966) found evidence for a fairly homogenous coating on the free surface of the endothelial cells of freshly enucleated eyes from the rhesus monkey. This material appeared to lose its bonding properties in direct proportion to the storage time, and after freezing and thawing of the cells, was readily washed from the endothelial surface by hypotonic saline. As the material was washed from the endothelium, it persisted longest over the intercellular spaces.

Using oblique illumination of methylmetacrylate replicas of the corneal surface, Wolf (1968) found evidence for a surface coating of "viscous material" on the endothelium of the macaque. Application of hyaluronidase did not produce a disappearance of the surface coat, suggesting that hyaluronic acid was not an important constituent. Wolf was, however, successful in removing the viscous material by repeated stripping of replicas from the same area. Following this process, he observed that, from the intercellular spaces, intercellular ledges of amorphous material stood out in relief. Stripping of the surface coat also revealed that each endothelial cell possessed a central flagella. A second means of removing the coating of viscous material was through the action of natural enzymes in the aqueous during prolonged storage at body temperature. Wolf found this viscous material impossible to stain with conventional methods.

MATERIALS AND METHODS

SELECTION OF SUBJECTS

Corneas were obtained from healthy domestic cats weighing 1.4 to 4.2 kg., with a mean weight of about 3 kg.

MEASUREMENT OF TEMPERATURE REVERSAL EFFECT

The cats were sacrificed by intracardiac injection of 50 mg/kg of pentobarbital. The eyes were enucleated immediately and washed with a polymyxin-neomycin ophthalmic solution.

The corneal thickness was then measured after the method of Maurice and Giardini (1950), using a Haag-Streit pachymeter attached to a Haag-Streit-AG-900 slit lamp with the light source locked at an angle of 35° to the biomicroscope. Six readings were made on each cornea, and the arithmetic mean calculated. The eyes were then sealed in enucleation jars with a saline-soaked gauze to maintain a moist atmosphere, and stored at 4°C for intervals of 48, 54, 58, 70, 76, 94, 114, 116, or 118 hours. Following storage, the corneal thickness was again measured. The eye was then placed in a small chamber with its posterior pole immersed in Eagles maintenance medium*, a complete cell culture medium. Via a small cannula, oxygen was bubbled through the medium into the incubation chamber while the eye was incubated for 2 hours at 37°C. Six measurements of corneal thickness were again taken and their arithmetic mean calculated. The temperature reversal -- the degree

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*Eagles Maintenance Medium - Department of Virology, Provincial Laboratory, Edmonton, Alberta.

to which warming reverses cold-induced swelling of the cornea -- was then calculated in terms of the increase in the thickness produced by storage:

$$T.R. = \frac{\text{Decrease in thickness with incubation}}{\text{Increase in thickness with storage}} \times 100$$

LISSAMINE GREEN STAINING

Lissamine green* is a non-toxic, anionic triphenylmethane dye. Its color index numbers are 737 (Society of Dyers and Colourists) and 44090. The L.G. powder was dissolved in normal saline to form a 1% solution of L.G. by weight.

Following temperature reversal measurements, a 6 mm. button was trephined from the center of the cornea and placed endothelial side up on a microscope slide. The 1% solution of L.G. was gently applied, allowed to remain on the endothelium for 20 minutes, an interval recommended by Holmberg (1961) and used by Jans and Hassard (1969); the stain was then blotted off without touching the endothelium. The corneal disc was precisely centered under the microscope and the endothelial surface photographed at x100. Around this central photograph, 8 more photographs were taken at equal distances in a uniform square pattern. Actual cell counts made in this lab by Jans and Hassard (1969) on x100 photographs using the identical Leitz Laborlux microscope with Orthomat camera showed an average of 2023 cells per photographic field.

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*Lissamine Green - Esbe Pharmaceutical Supplies, Toronto, Ontario.

INVESTIGATION OF THE SURFACE COAT

After finding evidence for an extraneous coating on the free surface of the endothelium, preliminary attempts were made to determine some of its characteristics by application of aqueous solutions of varying temperature and pH, organic solvents such as ethanol and glycol, and stains such as Periodic Acid Schiff, Alcian blue, Alcian red, and ninhydrin. These methods are described in a later section of this study.

RESULTS

Table I shows the temperature reversal obtained after varying intervals of storage, expressed in terms of the percentage reversal by heat of the increase in thickness produced by storage. Figure 1 shows the temperature reversal as a graph. The marked spread of values reflects the technical difficulties in pachymetry produced by rapid drying during measurement, stromal irregularities, and inexact centering of the cornea. The overall decline in temperature reversal with increased storage time, however, was apparent.

After storage for over 100 hours, most of the cat corneas had swollen to a thickness which could not be measured on the pachymeter scale, and hence, samples were small.

Table II shows the relation between the storage time and the number of stained cells. Figure 2 shows this relation as a graph. Jans and Hassard (1969) found a rough correlation between the length of storage and the number of cells stained with L.G. The curve relating cell staining to storage time showed a marked rise over the first 24 hours, and then a leveling off as storage time approached 48 hours. Our finding was that with storage time increased from 50 to 120 hours, not only was there no increase in the number of dead cells stained with lissamine green, but, because of increased skill and improved technique in removing corneas, the number of stained cells was actually smaller in the corneas stored for longer periods.

If it is postulated that in the freshly-enucleated eye the endothelial surface is covered and shielded from the stain by a surface coating, then two factors would determine the number of cells

stained. One would be the number of dead cells and the other, the integrity of the surface coating. Jans' finding of an increase in the number of dead cells in the first 48 hours would reflect the fact that during this period, some of the cells were still alive. After 50 hours, when presumably most, or all, of the cells were dead, the only factor determining the number of cells stained would be the intactness of the surface coat, and therefore the skill in avoiding trauma to the surface. The relatively high cell counts obtained early in the study -- at 50 and 62 hours -- reflect difficulty in avoiding the slight pressure or bending which so readily traumatized and caused staining of large areas of the endothelium. The low stained cell counts obtained in spite of extreme edema at 120 hours reflect marked improvement in technique, and probably give a more accurate picture of lissamine green staining of the untraumatized cornea after prolonged storage.

TABLE I

Temperature Reversal

Storage time in hours	50	56	62	74	80	98	118	122
Number of eyes	13	13	4	6	2	3	2	1
Mean temperature reversal	12	13	10	11	3.8	3.7	4.5	Nil
Standard error of means	2.9	4.5	1.6	1.4	0.94	1.8	0.84	Nil

TABLE II

Mean Cell Counts Following Storage

Time in hours following enucleation	50	56	62	74	98	120
Number of corneas	8	14	3	6	6	7
Mean cell count per 0.36 mm^2	86.6	55.3	87	57	55	18
Standard error of means	8.6	7.8	17	13	4.7	3.2
% of total cells possible per .36 mm^2	4.33	2.77	4.35	2.85	2.75	0.90

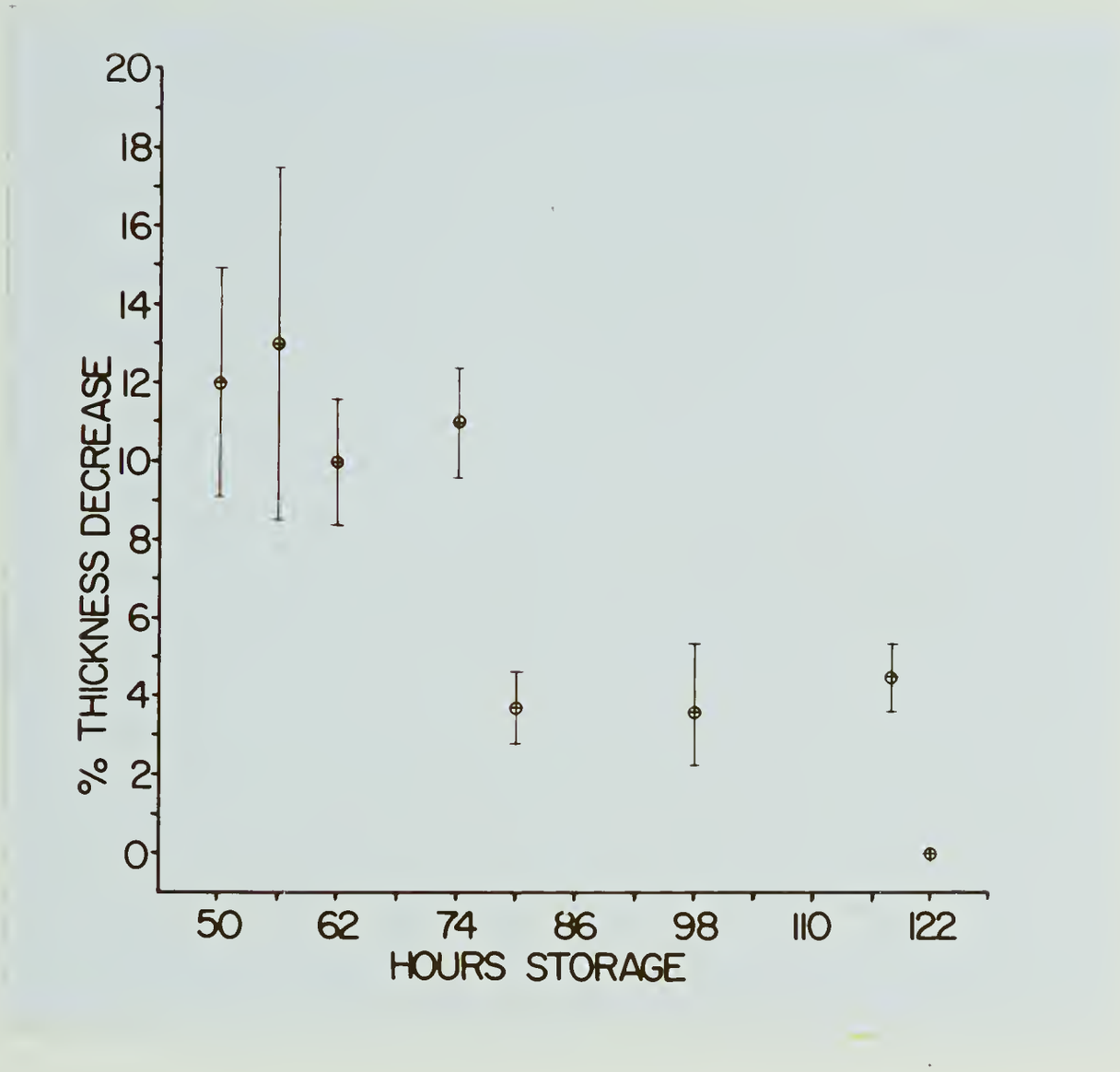

Figure 1. TEMPERATURE REVERSAL EFFECT. Graph shows the overall decline in temperature reversal effect in cat corneas with increasing storage time.

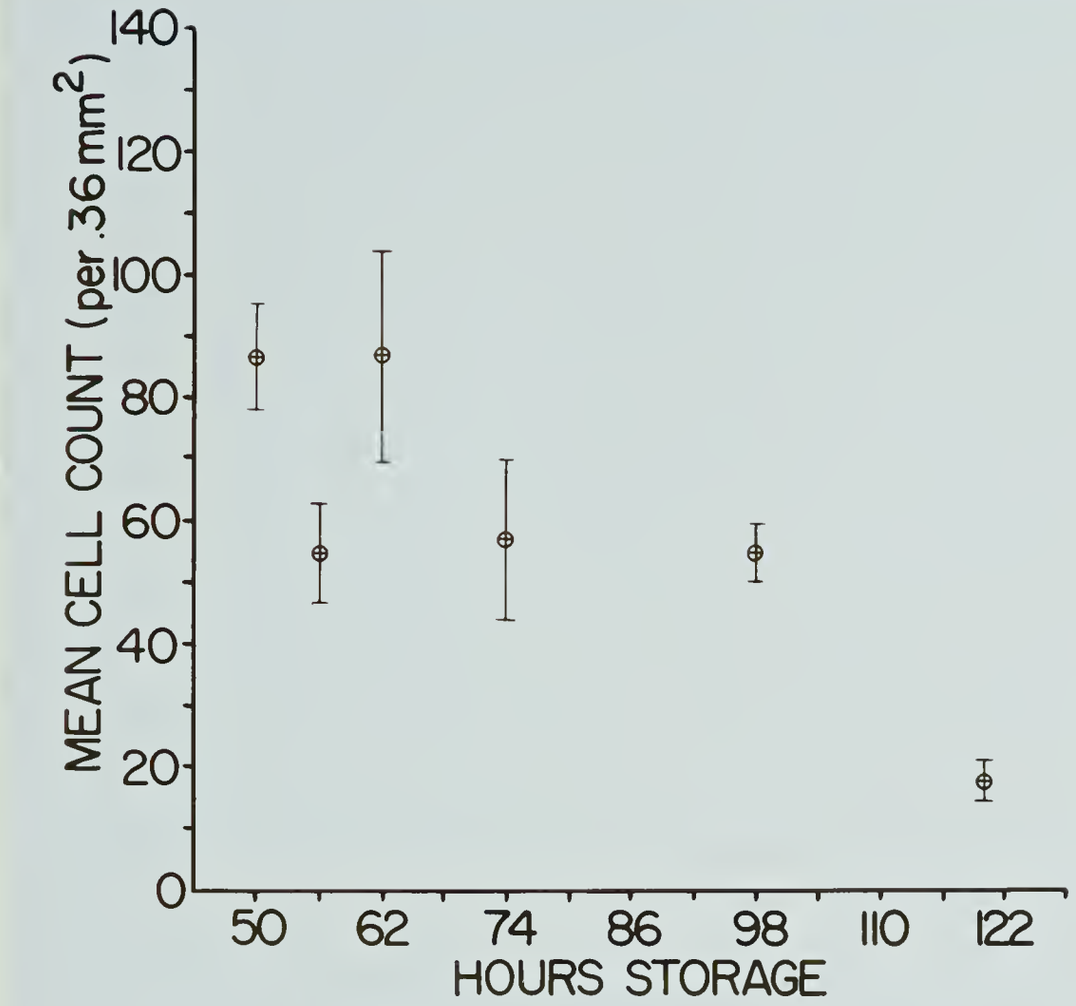

Figure 2. LISSAMINE GREEN STAINING. Graph shows that with increasing storage time, not only was there no increase in number of cells stained, but, with increased skill in preventing trauma, there was a marked decrease in cells stained.

DISCUSSION AND FURTHER INVESTIGATION

Our count of 86.6 dead cells stained out of a possible 2,000 per photograph corresponds closely to the count of 99 reported by Jans and Hassard (1969). In view of the relatively poor results obtained with full-thickness grafts in humans from corneas stored over 48 hours, we had expected more than 5% of the cells to be stained after 50 hours. Increasing the storage time up to 120 hours, we were surprized to find no increase in the number of dead cells staining with L.G. Since we had expected all the cells to be stained, we concluded that this aqueous supravital stain was not reaching the majority of the endothelial cells. This was the first of 3 findings which led us to postulate that the endothelial surface is covered by an extraneous surface coating.

The first finding -- that the number of cells staining with lissamine green was not increased by extremely prolonged storage -- suggests that, although the cells were dead, they were "waterproofed" by a free surface coating analogous to that demonstrated on the free surface of a variety of epithelial and non-epithelial cells elsewhere in the body.

The second finding was that cells which did stain were not uniformly distributed over the surface, but were most commonly found in irregular, linear groups over folds in the cornea (Fig. 3), suggesting that these cells were exposed to the stain where the surface coat was parted by the trauma of handling.

The third finding was made accidentally when a few drops of normal saline were placed on the endothelium of an L.G.-stained

Figure 3. L.G. staining of endothelial cells on a cat cornea stored 98 hours, when presumably the entire endothelium is dead. The cells were stained only in linear groups where the surface coating was parted by the trauma of folding. Note trailing processes of surface material along the retracted edges of the surface coat. (x100).

cornea. Instantly, the entire surface was covered by a dazzling mosaic of brilliantly refractile hyaline granulations, as if the saline had broken up a clear surface coating. The same effect was produced by hypotonic saline on fresh, unstained corneas, indicating that the surface coating was not produced by storage. These granulations stood out in relief with incident light, as well as transmitted light. We postulated that these granulations were formed by heaping up of the clear surface material of the surface coat. This heaping up involved only a partial thickness of the surface coat, because L.G. applied to the endothelial surface in its granular state failed to penetrate through to the underlying cells, which still remained unstained.

Surface patterns of the surface coat

The appearance of the endothelial surface under aqueous solutions fell into 5 patterns (Fig. 4) which we interpreted as changes in the state of the surface coating. These patterns were:

1) Unbroken surface
2) Mosaic of granules
3) Pericellular network of granules
4) Centricellular granules
5) Loss of the surface coat

First, on the untraumatized cornea, the surface was unbroken and free from stained cells. The surface layer was presumed to be intact and therefore invisible. The second pattern, the mosaic of granules, appeared upon application of normal saline to a cornea stored over 60 hours or of hypotonic saline to a fresh or stored

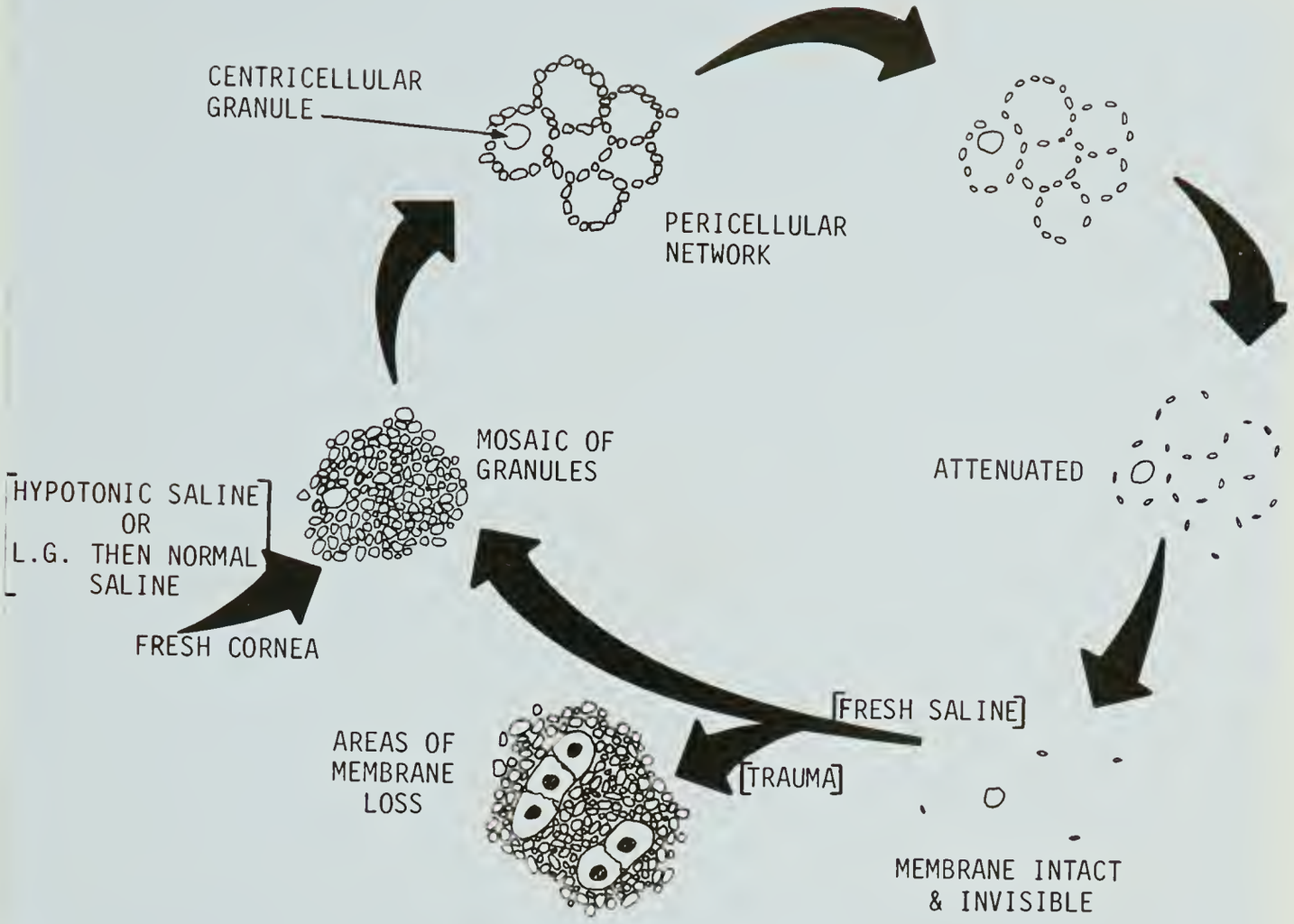

Figure 4. EFFECT OF HYPOTONIC SALINE ON ENDOTHELIAL SURFACE

Diagram shows changes in the appearance of the endothelial surface under a saline layer. These presumably reflect changes in the macromolecular configuration of the surface layer.

cornea. For one minute after application of the saline, the entire surface was completely covered by a mosaic of granules of varying size. Within 2 minutes, many of the granules disappeared, and nearly all those remaining lay over the intercellular spaces forming a pericellular network of granules (Figs. 5,6). If the cornea remained covered by saline, the net gradually became attenuated as these pericellular granules slowly shrank and disappeared (Figs. 7,8) and within 6 to 8 minutes, all the granules had disappeared, with the exception of a scattered few. After the granules disappeared while under saline, the surface material, though invisible, remained bonded to the cells, apparently having reverted back to an intact, invisible coating. Application of fresh saline caused the instantaneous reappearance of the same granular network, and after 5 minutes, again disappeared. This could be reqeated 6 times or more, but with increasingly large areas denuded of granules by the trauma of application and aspiration of saline. Addition of a few drops of fresh saline to a saline layer under which the granules had already disappeared caused a brief reappearance of the granules--for 1 to 3 minutes.

After the granules had disappeared while under the saline layer, if the same saline was removed and reapplied, the granules failed to reappear. This suggested that some of the ions from the saline were bound to the heaped up surface material as it reverted back to its smooth invisible state.

The third surface pattern, centricellular granules (Fig.9) appeared when the endothelium was subjected to chemical trauma such as acidic or dilute glycol solutions or to deionized water. These

Figure 5. PERICELLULAR NETWORK OF GRANULES. The hyaline granules persisting over the intercellular spaces are here focussed to appear as dark masses (x100).

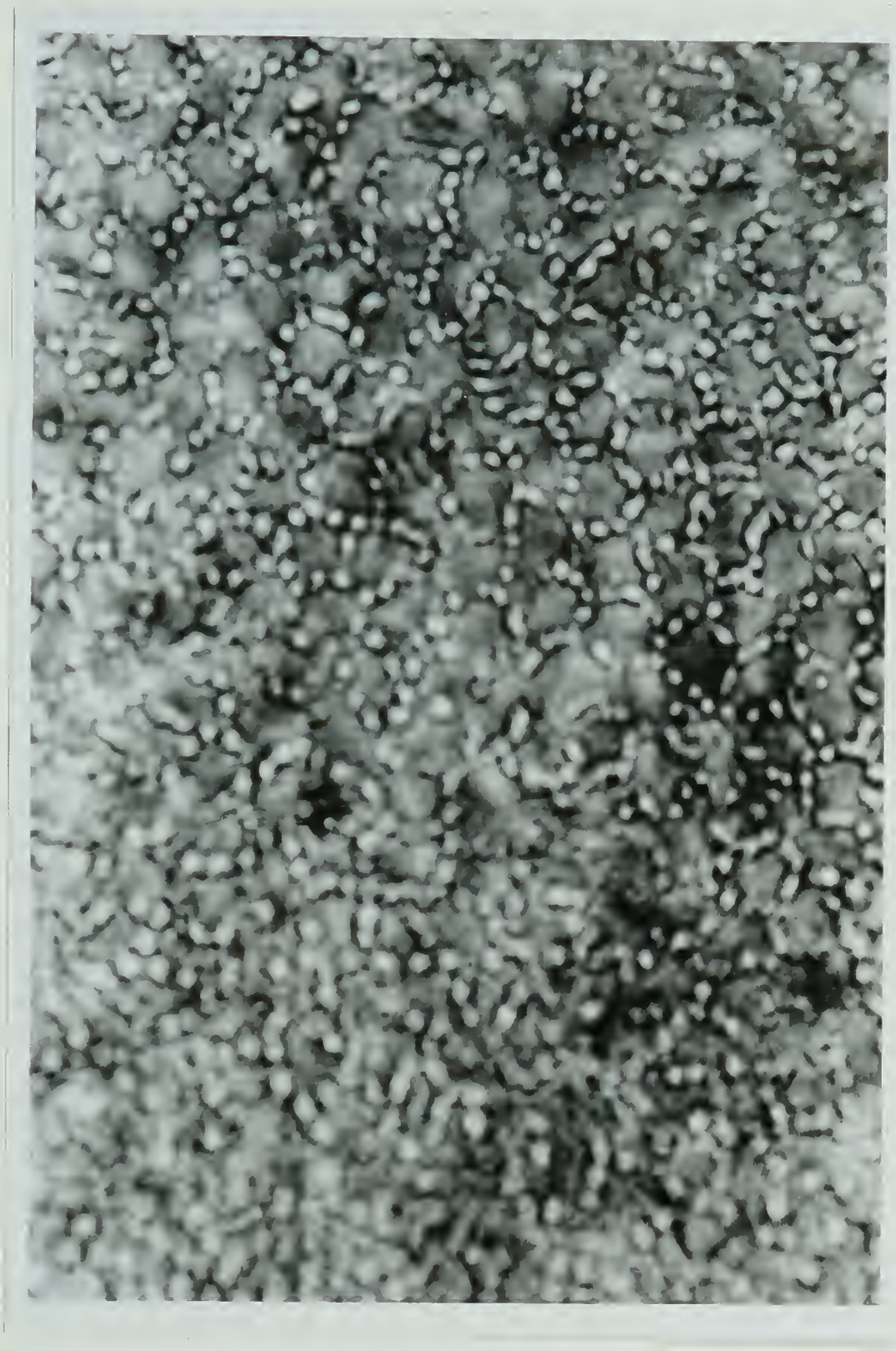

Figure 6. PERICELLULAR NETWORK. The endothelial cell borders are outlined by transluscent granules of varying size and shape. (x100).

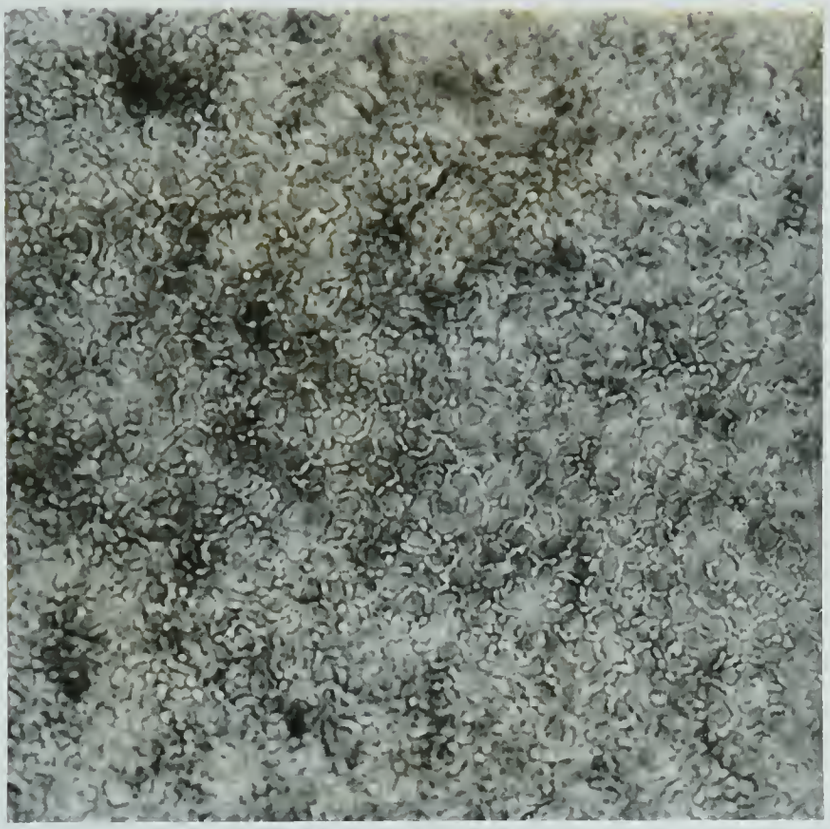

Figure 7. PERICELLULAR NETWORK. The granules show marked elongation along the axes of the intercellular spaces, suggesting interaction with the intercellular cement (x100).

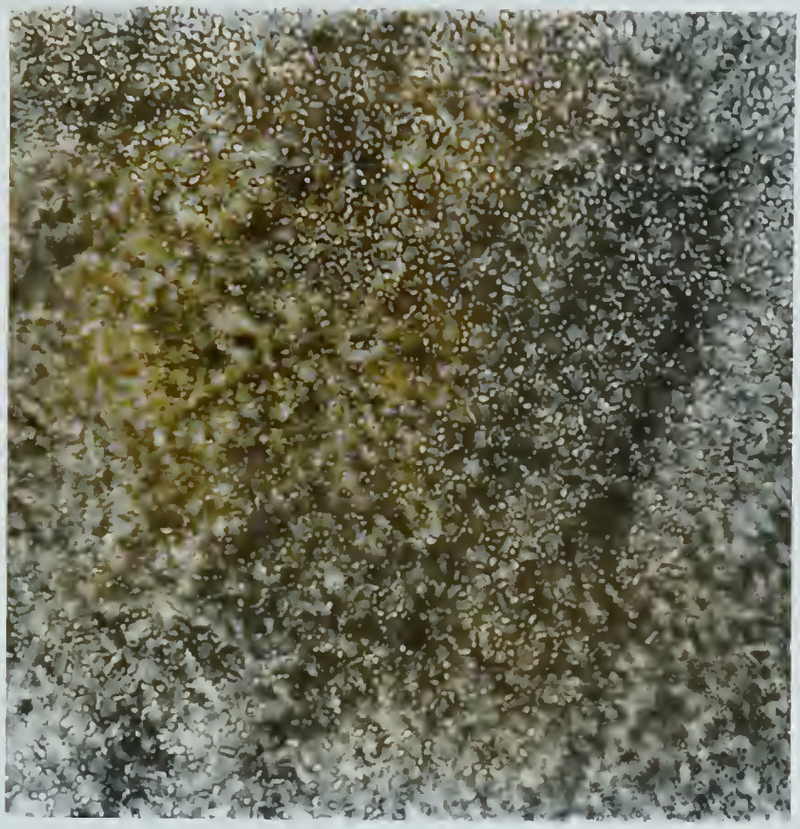

Figure 8. ATTENUATED PERICELLULAR NETWORK. From top to bottom, increasing attenuation is seen as granules grow smaller and disappear while under the saline layer. (x100).

Figure 9. CENTRICELLULAR GRANULES. These large, transluscent granules were produced on the endothelium by saline at pH 4.5. Some areas have been denuded of membrane and show 100% staining with L.G. (Stained cells show black nuclei). (x100).

relatively large granules lying over the centers of the cells failed to disappear when covered by electrolyte solutions. We suggest that they represent irreversibly denatured material derived from the surface coat.

The fourth pattern, loss of the surface layer, apparently required direct trauma to the surface in addition to death of the cells. For example, corneas stored for periods presumed long enough to kill the cells, required additional trauma such as folding or repeated flushing with saline to produce patches denuded of granules and therefore exposed to the lissamine green stain. In these circumscribed denuded areas, the cells showed gross morphological changes such as curling of their edges and 100% staining with lissamine green.

FACTORS INFLUENCING SURFACE GRANULARITY

Storage

Prolonged storage was the first factor found to predispose the endothelial surface to the granule-producing effect of aqueous solutions. Although hypotonic saline produced granularity on a fresh cornea, normal saline applied to a fresh cornea had no effect. Applied to a cornea stored over 60 hours, however, normal saline produced the typical dense, granular mosaic, suggesting that, with prolonged storage, enzymes in the aqueous or other factors reduced the cohesiveness of, or partially broke down, the macromolecular chains of the surface coat material.

Pretreatment with lissamine green

A second factor predisposing the surface to granule formation by electrolyte solutions was pretreatment of the surface with L.G. (and possibly with other large anions). A 1% solution of L.G. in normal saline had no apparent effect on a fresh cornea. Applied to a cornea stored over 60 hours, 1% L.G. in normal saline produced, not the typical dense granularity produced by saline lacking the 1% L.G., but only a few scattered granules. This suggests that at 1% concentration, the large, negatively-charged L.G. ion had a stabilizing effect on the surface. At the same time, however, preliminary application of 1% L.G. sensitized the surface to the granule-producing effect of subsequently-applied normal saline, a dense mosaic of granules appeared, even on fresh corneas, which were normally unaffected by either normal saline or L.G. alone.

Osmolarity

The third factor influencing surface granularity was the tonicity of the overlying electrolyte solution. Decreasing their tonicity below normal proportionately increased the effectiveness of electrolyte solutions in inducing surface granularity. Electrolyte solutions of less than half-normality invariably induced surface granularity in fresh corneas (which were unaffected by solutions of normal osmolarity). Decreasing the tonicity below normal also proportionately decreased the rate at which the granular mosaic reverted back through the pericellular network to an invisible,

intact surface. As the normality approached zero, an increasing number of centricellular granules were formed, and the few pericellular granules formed disappeared only incompletely over several hours. Deionized water produced a mosaic which never disappeared.

On the other hand, increasing the tonicity of the overlying electrolyte solution proportionately decreased its effectiveness in inducing granularity even in stored corneas. A 10 molar solution of sodium chloride failed to elicit surface granularity even in corneas stored 100 hours. Hypertonic solutions not only stabilized the surface coat, but increased the rate at which the granularity resolved. On corneas stored over 90 hours, 6-8 normal solutions produced dense granularity which disappeared in about 30 seconds or less, in contrast to the 6 to 8 minutes required under normal saline, and the more than 60 minutes under hypotonic solutions.

Temperature

Warming the saline by stages up to 45°C failed to alter the rate of disappearance of the pericellular granules.

pH

Solutions of pH 5 to 9 produced effects similar to those of normal saline. As the pH exceeded or dropped below this range, however, increasing numbers of irreversible centricellular granules formed, suggesting denaturation of the surface material.

SOLUBILITY OF GRANULES

To rule out the possibility that the granules actually dissolve in saline, saline was aspirated from the endothelial surface of 24 corneas after the granules had disappeared. Protein electrophoresis showed the presence in the saline aspirate of the identical protein band found in the normal aqueous humor -- a protein that migrates more rapidly than albumin -- possibly related to orosomucoid, but no identifiable with sera available to us. Most or all of the protein present in the saline aspirate was undoubtedly from aqueous contamination. Failure to find any protein or glycoprotein differing from that in the aqueous suggests that the granular surface material, if protein, is either the same protein as is normally present in the aqueous, or, more likely, that the granules do not dissolve in the saline when it reverts to the invisible state.

The pericellular granules were rapidly soluble in 5% ethanol, 5% glycol, and other organic solvents, leaving the cells permeable to L.G. We were unsuccessful in attempting to dissolve the granules without disrupting the cells. The centricellular granules, on the other hand, were resistant to being dissolved by dilute glycol or ethanol, a finding which supports the suggestion that they consist of denatured material.

STAINING

Since the surface coat remains heaped up into granules only under hypotonic solutions or after denaturation, and since few staining procedures allow the use of hypotonic solutions, it was extremely difficult to characterize the granules by staining techniques. In most cases,

following staining procedures, no granules could be found, but occasionally, one or two centricellular granules were seen. These granules showed great resistance to staining. Ninhydrin produced a purple color, indicating the presence of protein or glycoprotein. Periodic acid Schiff staining produced a very pale pink color, as did Alcian red. Alcian blue produced a very light blue color. Other stains, including fat stains left no evidence of surface granules. All staining techniques which required repeated flushing of the endothelium or non-aqueous fixatives or stains caused permanent disappearance of the pericellular granules, a factor which explains why this surface coat has not been previously demonstrated by conventional staining techniques.

SIMULATION OF CORNEAL SURFACE TO RULE OUT ARTIFACT

To determine if the hyaline granules were a physical artifact, attempt was made to simulate the endothelial surface with a monolayer of HeLa cells grown on the concave surfaces of contact lenses. Application of deionized water to this HeLa cell monolayer produced no evidence of hyaline granules.

SURFACE COAT IN HUMAN AND STEER EYES:

On the other hand, application of hypotonic saline to the corneas of human and steer eyes produced refractile granules with the same characteristics as those found in cat eyes. On human corneas the hyaline granules were considerably smaller than on cat corneas, suggesting that the surface coat is thinner. The granules were even finer in steer eyes than in human eyes. The mosaic of granules produced in human and steer eyes formed a pericellular

network and disappeared within the same time periods as in cat eyes.

In a sample of 8 human eyes stored for 56 to 74 hours, the numbers of dead cells stained with L.G. were larger than in cats' eyes stored for the same periods, but far below the numbers of dead cells expected. Three human eyes stored for 54 hours had counts of 45, 87 and 369 stained cells. Two stored for 44 hours had counts of 53 and 114. One stored for 68 hours had a cell count of 682. Two stored for 74 hours had cell counts of 311 and 840. The greater number of cells stained in human eyes stored for the same periods as the cat eyes also suggests that the surface coat is thinner and more fragile in human eyes.

MECHANISM OF GRANULE PRODUCTION AND DISAPPEARANCE

Assuming that, like free surface coatings elsewhere, the surface coat on the endothelium consists of glycoprotein, induction of surface granularity could be explained as follows: Glycoprotein consists of a complex of long, flexible chains with many fixed negative charges. The tendency of these chains to mutual repulsion is checked by the effect of counterions such as sodium which bind to, and cover, the fixed charges, allowing the acid polysaccharide chains to adopt their "relaxed" compact, coiled state. When, however, the glycoprotein surface layer is covered by a hypotonic aqueous solution, the counterions move out of the glycoprotein complex along the osmotic gradient. With their covering cations gone, repulsion occurs between the fixed charges on the polysaccharide chains and the chains repel each other and become rigid, forming granular areas

of heaped-up, "extended" chains. As long as the hypotonic solution remains, the surface remains granular. When the hypotonic solution is replaced by normal saline, sodium ions return to the polysaccharide chains, and, with their fixed charges covered, the molecules return to their compact, coiled form, and the surface again becomes unbroken.

SUMMARY AND CONCLUSIONS

Though it may be a reliable indicator or cell death, and though it showed an increase in the number of cells stained during the first 48 hours when some of the cells were still viable (Jans and Hassard 1969), lissamine green staining is not a useful test of corneal viability, as it fails to show any further increase in the number of dead cells stained in the endothelium of corneas stored from 48 up to 120 hours, when all the cells were presumed dead. This was the first of three findings which led to the hypothesis that the endothelial surface is covered by a surface coat. The second was that the stained cells were usually found in groups overlying folds in the cornea, suggesting that they were exposed to the stain where the surface coat was parted by trauma. After death of all the cells, the number of cells staining with lissamine green depends solely upon the degree of trauma to the surface coat

The third finding suggesting the presence on the endothelium of a surface coat was that application of hypotonic saline to the endothelial surface caused the appearance of a mosaic of refractile hyaline granulations which, under saline or other aqueous electrolyte solutions, gradually disappeared, forming first a pericellular network of granules, then an unbroken surface. The rate of disappearance of the granules was directly proportional to the tonicity of the electrolyte solution. Deionized water produced granularity of the surface of even fresh corneas, and the granules failed to disappear until the deionized water was replaced by saline. Prolonged storage or pretreatment of the endothelium with lissamine green--a large anionic molecule--predisposed the

surface coat to granulation by normal or hypertonic saline solutions which had no effect on fresh corneas. Chemical trauma, such as that produced by solutions of very low pH, caused the appearance of large, irreversible centricellular granules, which probably represent denatured material.

The refractile granules induced on the endothelium of human and steer corneas were finer than on cat corneas, suggesting that in the former, the surface coat is thinner. These granules were extremely difficult to stain, but staining characteristics, though very inconclusive, suggest that the granules may be glycoprotein.

Glycoprotein surface layers have been demonstrated on the free surface of a wide variety of cells. The free surface coat, together with the intercellular substance and the basal lamina, have been termed the glycocalyx, a glycoprotein investing coat postulated to be a feature of most mammalian cells (Bennet 1963). The fact that the surface granules persisted longest over the intercellular areas suggests an interaction and possibly a continuity between the intercellular cement and the surface coat on the endothelium.

Properties of the surface coat found on the endothelium can best be explained by assuming it to be a typical glycoprotein free surface coat. The production of surface granularity can be explained by the fact that glycoproteins consist of a complex of long, flexible chains with many fixed negative charges. The tendency of these chains to mutual repulsion is checked by cations which cover the fixed charges, allowing the polysaccharide complex to assume its compact, coiled state. Hypotonic solutions, however, set up an osmotic gradient which draws the positive counterions from the complex, so that the chains become

rigid and repel each other, forming tangled clumps of extended, linear molecules visible as surface granules. If the solution is only mildly hypotonic, sufficient cations could be rebound to the fixed charges on the polysaccharide chains to reduce repulsive interaction to a level where the surface continuity could be restored, possibly with a slightly different configuration of the polysaccharide chains. When deionized water is applied to the surface, however, no resolution of granularity occurs. On the other hand, increasingly hypertonic solutions produce increasingly rapid restoration of surface continuity.

It seems likely that, as in other organs, the glycoprotein surface layer on the corneal endothelium will be proved to function as a site for concentration of specific molecules for transport into the cell by pinocytosis, and, as such, may have a vital role in the nutrition of the endothelial layer, and, perhaps, of the stroma.

Further investigation of this surface coat should include disruption of the endothelium with an ultrasonic probe under deionized water, followed by ultracentrifugation of the cell particles in a hypotonic solution in the hope of isolating fragments of the surface coat in a granular state for chemical analysis and fluorescein antibody studies. Electron microscopy should also be done on the corneal endothelium using ruthenium red, osmium tetroxide, phosphotungstic acid, and other staining techniques which have been used successfully to demonstrate the free surface coat on the capillary endothelium and other tissues (Luft 1966, Groniowski et al 1969, and Pease 1966).

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